A 10-year retrospective comparison of two target sequences, REP-529 and B1, for Toxoplasma gondii detection by quantitative PCR

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A 10-year retrospective comparison of two target sequences, REP-529 and B1, for *Toxoplasma gondii* detection by quantitative PCR

Running title: REP-529 in clinical diagnosis

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Key-points (40 words): Few studies analyzed the impact of the lower sensitivity of B1-based quantitative PCR (qPCR) for the molecular diagnosis of toxoplasmosis. Our results show that B1 qPCR amplified only 54.4% of REP-529-positive clinical samples prospectively obtained from patients with toxoplasmosis.

Key words: diagnosis, toxoplasmosis, qPCR, Toxoplasma B1, Toxoplasma REP-529

Abstract

This study aimed to evaluate the repeated sequence REP-529 compared to the B1 gene for the molecular diagnosis of toxoplasmosis by quantitative PCR (qPCR) in routine diagnosis.

Over a ten-year period (2003-2013), all patients prospectively diagnosed with a positive REP-529 qPCR result for toxoplasmosis were included. All DNA samples (76 samples from 56 patients) were simultaneously tested using the two qPCR methods (REP-529 and B1).

The mean Ct obtained with the B1 qPCR was significantly higher (+4.71 cycles) than that obtained with REP-529 qPCR ($p<0.0001$). Thirty-one out of 69 extracts (45.6%) positive with REP-529 qPCR were not amplified with the B1 qPCR (relative sensitivity 54.4%, compared to REP-529), yielding false negative results on 15/28 placentas, 5 cord blood, 2 amniotic fluids, 4 cerebrospinal fluids, 1 aqueous humor, 2 lymph node punctures and 1 abortion product. This defect in sensitivity would have left 20/56 patients undiagnosed, distributed as follows: 12/40 congenital toxoplasmosis, 4/5 cerebral toxoplasmosis, 2/8 patients with retinochoroiditis, and both patients with chronic lymphadenopathy. This poor performance of B1 qPCR could be related to low parasite loads, since the mean *Toxoplasma* quantification in extracts with B1 false negative results was 0.4 parasite/reaction.

These results clearly show the superiority of the REP-529 sequence in the diagnosis of toxoplasmosis by PCR suggest that this target should be adopted as part of standardization of the PCR assay.

Introduction

Toxoplasmosis is a worldwide parasitic infection due to the intracellular parasite *Toxoplasma gondii.* The infection is usually asymptomatic in immunocompetent patients, and more rarely result in fever, lymphadenopathy or retinochoroiditis. By contrast, immunocompromised patients can experiment severe neurologic, ocular, pulmonary or disseminated disease (1). Yet toxoplasmosis is well-known for its pathogenicity during pregnancy. Indeed, when primary infection occurs in pregnant women, it can lead to congenital toxoplasmosis, with a frequency of transmission and a severity of fetal infection depending on the stage of pregnancy at which infection occurs (2). Diagnosis of toxoplasmosis is routinely based on serology. In some countries, such as France, seronegative pregnant women are monitored monthly by serology. In case of seroconversion, detection of *Toxoplasma gondii* DNA by PCR is a major diagnostic method for congenital toxoplasmosis, and is performed on amniotic fluid (prenatal diagnosis)(3-5), and placenta or cord blood at birth (postnatal diagnosis)(6-8). In immunocompromised patients, DNA can also be found in cerebro-spinal fluid (CSF), broncho-alveolar lavage (BAL) or others samples guided by clinical signs. The 35-fold repeated B1 gene (9) has been commonly used for this molecular diagnosis since 1989 with acceptable sensitivity (3, 5, 10), but another sequence (REP-529, Genbank AF146527) was described more recently, as being 200- to 300-fold repeated (11), leading to better detection of low parasite loads using spiked specimens (12). Our objective was to evaluate the diagnostic gain resulting from the routine use of a quantitative PCR (qPCR) targeting REP-529, compared to a qPCR targeting the B1 gene, for the diagnosis of toxoplasmosis in various clinical settings. In this study, we analyzed retrospectively the qPCRs results of all *T. gondii*-positive DNA samples from patients who benefited from molecular diagnosis over a 10-year period.

Materials and methods

Patients and sample collection

During the study period (2003-2013), all patients with a diagnosis of congenital toxoplasmosis who benefited from a molecular diagnosis on at least one sample (amniotic fluid, placenta or cord blood) were included. Congenital toxoplasmosis was defined by a positive prenatal diagnosis for *Toxoplasma gondii* (molecular diagnosis and/or mouse inoculation) and/or serologic evidence of antibody synthesis by the newborn at birth or during the one-year serologic monitoring (specific IgM or IgA detected by ISAGA (BioMérieux, Marcy-l'Etoile, France) or neosynthesized IgG detected by western-blot (LD-Bio, Lyon, France)). During the study period, routine qPCR molecular diagnosis

relied on both gene targets (B1 and REP-529) from 2003-2005, then only REP-529 has been used until now. All positive samples were re-analyzed with both gene targets in the same qPCR run, to homogenize parasite quantification, as standard curve had changed meanwhile. Additionally, all samples from proven congenitally infected infants which had been tested negative in routine diagnosis with REP-529 were also retested in parallel with the B1 PCR.

Besides, adult patients with clinical signs compatible with toxoplasmosis (lymph nodes with specific anti-*Toxoplasma* IgM, uveitis, retinochoroiditis, cerebral abscess) and a positive DNA detection with REP-529 from 2009-2013 were also included, and DNA samples were re-analyzed with both qPCRs.

Molecular diagnosis

On reception of the placenta (PL), several samples were taken from different sites and mixed in a solution containing 2.5 mg/mL trypsin, penicillin 500 IU/mL, and gentamicin 3.3µg/mL. The preparation was digested for 2 hours at 37°C under agitation, filtered through gauze and centrifuged for 10 minutes at 1000 g. The supernatant was discarded and the pellet was washed 3 times. Then, 200µl of prepared placentas and other crushed biopsies (lymph-node or cerebral) were digested overnight with proteinase K at 56°C prior to extraction. Amniotic fluids (AF) samples (10mL) were centrifuged at 1500xg for 10 min then supernatant was discarded and 2x200µL samples of the pellet were used for DNA extraction. Other fluids (CSF, aqueous humor (AH)) were centrifuged at 1500xg for 10 min, and 200µL of pellet was used for DNA extraction. Routinely, DNA extraction of all samples was performed using QIAmp DNA mini kit® columns (Qiagen, Courtaboeuf, France) and eluted in 100µL, except for blood (peripheral blood (PB) and cord blood (CB)) samples, which were processed using 1-2 mL of whole blood and extracted using QIAamp DNA midi kit® columns (Qiagen), and eluted in 400 µL. Two extracts were performed for amniotic fluids and placentas (only one for other samples) and amplification was run in duplicate for all samples.

The primers and probe used to amplify a 98 pb fragment of the B1 gene were 5'-GAA AGC CAT GAG GCA CTC CA-3' (forward) and 5' –TTC ACC CGG ACC GTT TAG C- 3' (reverse), and FAM™-5'-CGG GCG AGT AGC ACC TGA GGA GAT ACA-3'-TAMRA™ (13), and primers and probe targeting REP-529 were those described previously (6). All samples were re-analyzed by both qPCRs in the same run, using the following conditions: 25µl reaction mixture containing primers and probe at a final concentration of 600 nM and 200 nM, respectively, 12.5 µL of TaqMan™ Universal PCR Master mix (Applied Biosystems, Courtaboeuf, France), and 5 µL of DNA sample. Amplification was performed on a Step One plus device (Applied Biosystems) for 40 cycles (15 sec 95°C and 1 min 60°C) preceded by 10 min at 55°C for UNG reaction and 10 min denaturation at 95°C. Appropriate controls were included in each run (positive and negative controls, internal control of inhibition). Positive samples were

quantified using standard curve obtained by serial dilutions of a standardized control (10^5 *Toxoplasma* RH strain), provided by the National Reference Center for Toxoplasmosis. In the aim to compare quantification data over the study period, all positive samples were re-analyzed for quantification using the same standard curve. The cycle threshold (Ct) of positive samples was recorded for comparison of REP-529 and B1 qPCRs. All Ct results previously acquired during routine analysis and results acquired after retesting for parasite quantification for the purpose of this study were compared to ensure that long storage did not alter DNA.

Statistical analysis

Before comparing the sensitivity of REP-529 and B1 targets, we verified the quality of DNA samples stored at -20°C, by using a Wilcoxon paired test to compare the Ct newly obtained with REP-529 qPCR to the Ct previously recorded in routine diagnosis.

For the statistical analysis of relative sensitivity between B1 and REP-529 qPCRs, paired Ct obtained with both qPCRs were compared using the Wilcoxon test. The mean Ct obtained with B1 or REP-529 qPCR per type of sample were also compared using the Mann-Whitney test. Only newly acquired quantitative results obtained with both PCR simultaneously were taken into account for statistical analysis. Absolute sensitivity of each PCR for the diagnosis of congenital toxoplasmosis was evaluated on the whole cohort of congenitally infected infants diagnosed over the study period who benefited either from prenatal diagnosis or neonatal diagnosis by PCR.

Statistical analysis was made using GraphPad® Prism V5 (GraphPad software, USA). $P < 0.05$ was considered significant.

Results

Patients and samples

Forty-one cases of congenital toxoplasmosis were included, consisting of 20 amniotic fluids, 1 abortion product, 35 placentas and 5 cord blood samples. Additionally, 7 cases of reactivation toxoplasmosis in immunocompromised patients (2 blood samples, 4 CSF, 1 vitreous fluid and 1 cerebral biopsy) and 7 cases of symptomatic toxoplasmosis in immunocompetent patients were included (2 lymph node biopsy specimens, 5 aqueous humors). Overall, 76 samples from 56 patients were analyzed. Clinical and biological data of the patients are detailed in Table 1.

Lack of impact of storage on qPCR Ct

Forty-nine samples were retrospectively re-analyzed with B1 qPCR in this work, thus it was essential to verify that long-term storage at -20°C after initial diagnosis had not altered DNA. The mean Ct of REP-529 qPCR obtained on samples before (at time of diagnosis) and after storage were similar (31.9 ± 0.8 versus 32.1 ± 0.8, p=0.8347). Additionally, the Ct results were compared using a Wilcoxon paired test, which confirmed that they did not differ (p = 0.1631, data not shown), thus making possible the interpretation of the data.

Comparison of B1 qPCR vs REP529 qPCR

The performance of both qPCR targets was analyzed on 76 samples. Overall, 31 of 69 extracts (45%) tested positive with REP-529 qPCR were not amplified by the B1 qPCR, thus the relative sensitivity of the B1 qPCR was 55%, compared to the REP-529 qPCR (Table 2). Seven false negative results (7 placenta samples) were also observed with REP-529; none was positive with the B1 qPCR. Thus, over the study period, the absolute sensitivity of REP-529 qPCR and B1 qPCR on placenta samples was 80% and 37%, respectively (Tables 1&3). Their absolute specificity for prenatal diagnosis was 100% and 90%, respectively (Table 3). False negative results with the B1 qPCR, compared to REP-529 qPCR, were observed on all sample types, yet mainly on placenta samples (15/28, 54%), blood samples (6/7, 86%), and CSF (4/4, 100%) (Table 2). Overall, the mean Ct obtained with REP-529 qPCR when B1 qPCR was negative was 36.84 ± 0.36 (corresponding to 0.37 ± 0.3 parasites/reaction), underlining the need for a very sensitive qPCR assay. For the 38 samples which could be amplified with both qPCRs, the mean Ct obtained with the B1 qPCR was significantly higher (+4.7 ± 0.3 Ct) than that obtained with REP-529 qPCR ($p<0.001$) (Table 4). The mean gain in amplification Ct ranged from 3.65 to 4.98 according to the type of sample, and was highly significant for amniotic fluids and placenta samples ($p<0.001$, Table 4). The difference was not statistically significant for aqueous humor samples, probably because of a lack of statistical power.

Regarding congenital toxoplasmosis, the B1 qPCR failed to amplify any of the available samples (AF, PL or CB) in 11 out of 40 cases (27.5%) which were positive with REP-529 qPCR. This defect in sensitivity would have had variable consequences according to the results of other biological techniques used. Importantly, prenatal diagnosis would have been falsely negative in two cases (#19 and 31), and fetal infection would have remained unproven in a case of fetal loss (#17). Of note, B1 target also yielded a false negative result on the placenta sample from case #19 (Table 1). In three other cases (#20, 26, 27), where no prenatal diagnosis had been performed because of late maternal infection during the third trimester of pregnancy, the B1 qPCR would have left the newborns undiagnosed, since no serological evidence of infection was observed until several months (Table 1). For the five remaining cases (#8, 11, 15, 22, 30), the consequences of the reduced sensitivity of B1

qPCR would have been negligible, as serological markers of congenital infection (specific IgM or IgA detection, neosynthesized IgG on western-blots) were observed in newborns at birth or during the first week of live. In case #15, B1 qPCR was falsely negative in both placenta and cord blood. In the remaining cases, the lack of sensitivity of B1 on placenta samples was moot, since the diagnosis had been made previously on AF.

Regarding ocular toxoplasmosis, one aqueous humor from an immunocompetent patient for whom Western blot was not contributive, and one blood sample from a HIV+ patient, who did not undergo aqueous humor puncture (Table 1), were negative with B1, which would have left 2 patients undiagnosed. Additionally, the B1 target would have left undiagnosed 4 out of 5 (80%) cerebral toxoplasmosis, and the two patients with chronic lymphadenopathy (Table 2).

Discussion

This study including all positive samples obtained by REP-529 qPCR over a 10-year period, clearly shows the superiority of the REP-529 target for the diagnosis of toxoplasmosis, whatever the clinical setting. As previously described in other studies, this can be explained by the difference in the number of repetitions of this sequence (about 7- to 10 fold more repeated than B1)(11), which results in a gain of about 4 C_t (12, 14), as observed here ($p<0.001$), and allows detection of 10-fold lower parasite loads (14). However, few studies evaluated the impact in terms of sensitivity of diagnosis in routine clinical use.

Filisetti et al. (15) compared three PCR methods on 23 selected AF samples and 16 CB from 19 cases of congenital toxoplasmosis, and found that only 5 out of 9 REP-529-positive AF were positive with the B1 PCR. It must be noted that the overall sensitivity in this study was very low, since only 11 out of 19 AF (58%) were positive with the reference method used, i.e. rDNA PCR (16), which could be due to the use of conventional PCR methods in this study. Cassaing et al. (14) included 33 positives samples (8 AF, 15 PL, 3 AH, 3 CSF, 2 blood samples and 2 BAL), of which 13/15 (87%) PL and 8/8 AF (100%) tested positive with REP-529 qPCR were also positive with B1 qPCR. No differences in sensitivity between both targets were observed for AH, CSF and BAL samples, but all samples were tested in duplicate and in 11 instances, negative results were observed in 1 of the 2 amplifications with B1 qPCR, whereas one sample was amplified by REP-529 qPCR only once. Additionally, the authors considered a B1 result as positive, although it was over 40 Ct, in 4 samples. Another study (17) included prospectively 135 AF samples, of which 27 and 22 were positive with REP-529 and B1 qPCR, respectively. The authors declared that 2 of the 5 presumed false negative B1 results were in fact false positive REP-529 results, however, no details are provided on the newborns follow-up and

the criteria that led them to this conclusion. Finally, the most recent study (18) evaluated three qPCR methods, mainly on AF samples. The authors reported 33 positive results with their B1 qPCR, compared to 43 with two REP-529 qPCR methods, leading to a relative sensitivity of 77% for B1. Two cord blood samples tested positive with REP-529 were negative with B1 qPCR. Additionally, they found that their two REP-529 qPCRs methods and devices (Applied Biosystem and Roche) performed equally.

Our study focused on the evaluation of the relative sensitivity of B1 versus REP-529 PCR targets, thus all PCR-positive samples were included. Additionally, all samples (AF, PL) from congenitally infected children with a negative REP-529 qPCR result were retested. No samples with B1-positive and REP-529-negative results were observed, either prospectively or retrospectively, in cases of proven congenital toxoplasmosis. The overall relative sensitivity of the B1 qPCR was only 55%, and its absolute sensitivity was 37% in the setting of congenital toxoplasmosis (Tables 2&3). This defect in sensitivity was particularly crucial for 3 antenatal diagnoses (2 prenatal diagnoses and 1 early fetal loss undiagnosed) and 3 neonatal diagnoses. In case of fetal loss, the recognition of the role of *Toxoplasma* is important, because it allows to eliminate other causes of spontaneous abortion and avoids useless investigations. On the other hand, the positivity of prenatal diagnosis usually leads to a change in chemotherapy, using pyrimethamine-sulfonamide combination therapy to treat the mother (1-2), which would have been missed in the two B1-negative patients here. Finally, the parasite DNA detection in placenta, even if not yet recognized as a standard criteria for the diagnosis of congenital toxoplasmosis, was shown to have a positive predictive value over 90% in our hands (6), and is at least a strong argument to accurately follow the infant and increase the frequency of serologic testing to confirm infection, with the aim of reducing delay of treatment. We observed here that parasite DNA detection from placenta was the earliest biological sign of infection in neonates with neither IgM nor IgA detection, and recall the interest of this sample in patients for whom antenatal diagnosis was negative or not performed. In 2 cases (#23, 41), both qPCR were negative on placenta samples, whereas they were positive with mouse inoculation, still underlining the interest to combine both techniques (Table 1).

The B1 qPCR had also poor sensitivity in other clinical settings. The diagnosis of cerebral toxoplasmosis is difficult, and ancient studies have reported a poor sensitivity of conventional PCR methods (19-20), probably related to low parasite loads, but no recent studies evaluated new qPCR methods in this setting. The present study was not designed to answer this question, but we noted that four patients would have been undiagnosed using B1 qPCR. Besides, the REP-529 qPCR allowed diagnosing two cases of chronic toxoplasmosis in patients with lymphadenopathy and history of

Toxoplasma seroconversion in the past twelve months, which led to consider this diagnosis and stop further exploration aiming at diagnosing a hematological malignancy.

False negative results targeting B1 had a C_t > 34 (mean 36.84 ± 0.36). This poor performance of B1 qPCR could be related to low parasite loads, a frequently observed situation in congenital toxoplasmosis, particularly in France where women are treated all along pregnancy, which probably decreases the parasite burden. In the study by Romand et al. (21), 88 positive AF were included, of which 35 (40%) were shown to contain less than 10 parasite/mL.

Besides, the proportion of undetected samples with the B1 qPCR was high for placenta samples (57% of REP-529-positive samples) and blood samples (50%). In 12 placenta samples, B1 qPCR was negative whereas mouse inoculation was positive, which is unusual. Poor sensitivity on blood and placenta samples suggests that inhibitors would more likely interfere with this PCR. Indeed, Chabbert et al. (22) nicely showed that the efficacy of PCR on samples spiked with low amounts of parasites was lower in placenta or blood, compared to AF, with both sets of B1 primers used.

After the description of the REP-529 sequence, the B1 target has been still frequently used in parallel in most labs, including ours, until it could be demonstrated that the occurrence of B1 positive results and REP-529 negative results was never observed (14-15, 18), suggesting that this sequence is present in all parasite isolates. However, a recent Brazilian study (23) reported a lower proportion of positive amniotic fluids with REP-529 target, than with B1 (36.5% and 87.3%, respectively), with only 23.8% of samples being positive with both targets. This unusual finding must be verified on other series of clinical samples from South America, to check if atypical parasite strains circulating in this area could lack the REP-529 sequence or have mutations or modification of the number of repetitions, that could lead to a decreased sensitivity of this PCR target, which now appears to be the gold standard for European parasite strains.

Therefore, in view of the results obtained in the present study, we suggest widespread use of the REP-529 qPCR target, which should replace B1 target, at least in Western countries, until its value is confirmed in South America.

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344 Table 1: Clinical characteristics and qPCR results for 41 mother-child pairs of congenital toxoplasmosis, 8 cases of ocular toxoplasmosis, 5 cases of cerebral
345 toxoplasmosis and 2 cases of chronic lymphadenopathy

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Case N°	Clinical setting	Clinical signs (Pregnancy trimester at maternal infection for CoT)	Sample	qPCR results		Mouse inoculation	Other biological criteria of infection
				B1	Rep-529		
1	CoT	Abnormal neurodevelopment (ultrasound) : Medical termination of pregnancy (T1)	AF	+	+	+	Positive qPCR (REP) on fetal biopsy
			PL	+	+	ND	
2	CoT	Asymptomatic (T3)	AF	+	+	+	IgM detection at 3month in the infant (in another hospital), PL positive by mouse inoculation
3	CoT	Asymptomatic (T3)	PL	+	+	+	Positive prenatal diagnostic in another hospital.
			CB	-	+	ND	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
4	CoT	Asymptomatic (T3)	PL	+	+	ND	Neosynthesized IgG/IgM by WB; IgM and IgA detection at 1 month
			CB	-	+	ND	
5	CoT	Asymptomatic (T3)	PL	+	+	+	IgM at birth
6	CoT	Asymptomatic (T2)	AF	+	+	-	Persisting IgG at 1 year of life
			PL	-	+	+	
7	CoT	Ventriculomegaly (ultrasound) : Medical termination of pregnancy (T1)	PL	+	+	+	*Toxoplasma* detection in AF by mouse inoculation
8	CoT	Asymptomatic (T3)	PL	-	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
9	CoT	Intracerebral calcifications (ultrasound) (T2-3)	AF	+	+	+	Persisting IgG at 1 year of life, *Toxoplasma* detection in PL by mouse inoculation
10	CoT	Asymptomatic (T2)	PL	+	+	+	Neosynthetized IgG by WB
11	CoT	Seizure after birth (T3)	PL	-	+	+	Positive prenatal diagnostic in another hospital,

							neosynthesized IgM (WB) at birth
12	CoT	Unknown (T3)	PL	+	+	+	Neosynthesized IgG/IgM by WB
13	CoT	Intrauterine fetal death (T2)	AF	+	+	+	
14	CoT	Asymptomatic (T3)	AF	+	+	+	Neosynthesized IgG/IgM, IgM and IgA detection at birth
			PL	-	+	+	
15	CoT	Asymptomatic (T3)	PL	-	+	-	IgM and IgA detection at 1 week of life
			CB	-	+	ND	
16	CoT	Unknown (T3)	PL	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at
			CB	-	+	ND	birth
17	CoT	Termination of pregnancy (T1)	AP	-	+	ND	
18	CoT	Chorioretinitis (T2)	AF	+	+	+	IgM detection at birth
			PL	-	-	-	
19	Co T	Asymptomatic (T3)	AF	-	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at
			PL	-	+	+	birth
20	CoT	Asymptomatic (T3)	PL	-	+	+	IgM (in another hospital) at birth
21	CoT	Asymptomatic (T3)	AF	+	+	+	Neosynthesized IgG/IgM by WB at birth
22	CoT	Asymptomatic (T3)	PL	-	+	+	Positive prenatal diagnostic in another hospital
23	CoT	Asymptomatic (T2)	AF	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at
			PL	-	-	+	birth
24	CoT	Chorioretinitis (T2)	AF	+	+	+	IgM and IgA detection at birth and neosynthesized
			PL	-	+	+	IgG/IgM by WB at 1 month
25	CoT	Asymptomatic (T1)	AF	+	+	+	
			PL	-	-	-	
26	CoT	Unknown (T3)	PL	-	+	+	Persisting IgG at 1 year of life
27	CoT	Asymptomatic (T3)	PL	-	+	+	Neosynthesized IgG by WB at 3 months of life
28	CoT	Chorioretinitis (T3)	AF	+	+	+	IgM and IgA detection at birth and neosynthesized IgG
			PL	-	+	+	(WB) at 1 month of life
29	CoT	Asymptomatic (T2)	AF	+	+	+	Neosynthesized IgG at 3 month (WB)

			PL	-	-	-	
30	CoT	Asymptomatic (T3)	PL	-	+	-	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
31	CoT	Asymptomatic (T2)	AF	-	+	-	DNA detection by REP-529 qPCR in cord blood (B1 not determined)
			PL	-	-	-	
32	CoT	Asymptomatic (T3)	PL	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
			CB	-	+	ND	
33	CoT	Asymptomatic (T3)	AF	+	+	+	IgM and IgA detection at birth, neosynthesized IgG by WB during follow-up
			PL	-	+	+	
34	CoT	Unknown (T3)	PL	+	+	ND	Positive prenatal diagnostic in another hospital
35	CoT	Chorioretinitis and intracerebral calcifications (T2)	AF	+	+	+	Persisting IgG at 1 year of life
			PL	-	-	-	
36	CoT	Asymptomatic (T3)	AF	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
			PL	-	+	-	
37	CoT	Asymptomatic (T3)	PL	+	+	+	Neosynthesized IgG/IgM by WB; IgM and IgA detection at birth
38	CoT	Asymptomatic (T3)	AF	+	+	+	
			PL	+	+	+	
39	CoT	Unknown (T3)	AF	+	+	+	Positive serological monitoring in another hospital
40	CoT	Asymptomatic (T3)	PL	+	+	+	Positive prenatal diagnostic in another hospital, IgM at birth
41	CoT	Asymptomatic (T3)	PL	-	-	+	IgM and IgA detection at birth and neosynthesized IgG (WB)
42	OT	Uveitis	AH	+	+	ND	
43	OT	Uveitis	AH	+	+	ND	Neosynthesized IgG in AH (WB)
44	OT	Uveitis and chorioretinitis	AH	+	+	ND	
45	OT	Uveitis	AH	+	+	ND	Neosynthesized IgG in AH (WB)
46	OT	Hyalitis, not treated for toxoplasmosis	AH	-	+	ND	

47	OT	Uveitis, HIV + patient. Treated for toxoplasmosis	VF	+	+	ND	Neosynthesized IgG in VF (WB)
48	OT	Uveitis, HIV + patient. Treated for toxoplasmosis	PB	-	+	ND	Serological reactivation (high levels of IgG and IgM)
49	OT	Retinochoroiditis, HIV+ patient. Treated for toxoplasmosis.	PB	+	+	ND	
50	CeT		CSF	-	+	ND	
51	CeT	Neurological symptoms, HIV+ patient	CSF	-	+	ND	
52	CeT	Immunocompromised patient, 5 cerebral lesions on CT scan	CeB	+	+	ND	DNA detection in CSF in another hospital
53	CeT	Heart transplant patient, neurological symptoms	CSF	-	+	ND	IgM detection in serum
54	CeT	Cerebellar syndrome	CSF	-	+	ND	Serological reactivation (high levels of IgG and IgM)
55	CL	Chronic toxoplasmosis, asthenia	LNP	-	+	ND	History of seroconversion (persisting IgM)
56	CL	Chronic toxoplasmosis, asthenia	LNP	-	+	ND	History of seroconversion 6 month before

CoT, congenital toxoplasmosis; AF, amniotic fluid; PL, placenta; CB, cord blood; AP, abortion product; T1, first trimester of pregnancy; T2, second trimester of pregnancy; T3, third trimester of pregnancy; OT, ocular toxoplasmosis; AH, aqueous humor; PB, peripheral blood; VF, vitreous fluid
CeT, cerebral toxoplasmosis; CSF, cerebro-spinal fluid, CeB, cerebral biopsy
CL, chronic lymphadenopathy; LNP,lymph node puncture; ND, not determined ; WB, Western-blot

353 Table 2: Relative sensitivity of B1 qPCR on samples tested positive with REP-529 qPCR, according to

354 the type of sample

Sample	B1 qPCR result No positive/Total No (%)
Amniotic fluid	18/20 (90)
Placenta	13/28 (46.4)
Abortion product	0/1 (0)
Cord blood	0/5 (0)
Blood	1/2 (50)
Ocular fluids	5/6 (83)
Cerebrospinal fluid	0/4 (0)
Biopsy (cerebral or lymph node)	1/3 (33)
All samples	38/69 (55)

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Table 3: Sensitivity of B1 qPCR and REP-529 qPCR for the diagnosis of congenital toxoplasmosis (2003-2013)

Sample	B1 qPCR result No positive/Total No (%)	REP-529 qPCR result No positive/Total No (%)
Prenatal diagnosis (amniotic fluid)	18/20 (90)	20/20 (100%)
Neonatal diagnosis (placenta)	13/35 (37)	28/35 (80%)

361 Table 4: Mean Cycle threshold (Ct) obtained with B1 qPCR and REP-529 qPCR, according to sample

362 type.

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Sample	Ct B1 (Mean ± SEM)	Ct Rep 529 (Mean ± SEM)	ΔCt (Mean ± SEM)	P value[a]	P value[b]
Amniotic fluid (n = 18)	34.80 ± 0.58	29.87 ± 0.57	4.93 ± 1.35	0.0002***	<0.0001***
Placenta (n = 12)	35.86 ± 0.57	30.87 ± 0.58	4.98 ± 2.07	0.0002***	<0.0001***
Ocular fluids (n =5)	32.98 ± 2.00	29.84 ± 2.30	3.49 ± 1.1	0.0625	0.3095
All samples (n = 37)	34.51 ± 0.56	29.7 ± 0.53	4.81 ± 0.31	<0.0001***	<0.0001

364 [a] Wilcoxon test; [b] Mann Whitney test

365 ***, p<0.001

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